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# **Development of Phenotypic and Transcriptional Biomarkers to Evaluate Relative Activity of Potentially Estrogenic Chemicals in Ovariectomized Mice**

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**Running title:** Activity of candidate xenoestrogens

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## Abstract

**Background:** Concerns regarding potential endocrine disrupting chemicals (EDCs) have led to a need for methods to evaluate candidate estrogenic chemicals. Our previous evaluations of two such EDCs revealed a response similar to that of estradiol (E<sub>2</sub>) at 2 hours, but a less robust response at 24 hours, similar to the short-acting estrogen, estriol (E<sub>3</sub>).

**Objectives:** Microarray analysis using tools to recognize patterns of response have been utilized in the cancer field to develop of biomarker panels of transcripts for diagnosis and selection of treatments most likely to be effective. Biological effects elicited by long vs. short-acting estrogens greatly impact the risks associated with exposures, therefore it is important to develop tools to predict the ability to maintain estrogenic responses.

**Methods:** Biological endpoints in uterine tissue and a signature pattern-recognizing tool that identified co-expressed transcripts allowed development and testing of a panel to classify potentially estrogenic compounds using an *in vivo* system. Although the endpoints are relevant to uterine tissue, the resulting classification of the compounds is important for other sensitive tissues and species.

**Results:** We evaluated biological and transcriptional endpoints with proven short and long-acting estrogens, and verify the use of our approach using a phytoestrogen. With our model, we were able to classify the diarylheptanoid D3 as a short-acting estrogen.

**Conclusions:** We have developed a panel of transcripts as biomarkers which, together with biological endpoints, might be used to screen and evaluate potentially estrogenic chemicals and infer mode of activity.

## Introduction

The ovariectomized mouse uterus exhibits rapid biochemical and biological responses to estrogens that have been extensively studied and characterized (Katzenellenbogen et al. 1979). Using this mouse model we previously evaluated transcript responses by microarray and have identified several thousand genes with transcripts that are increased or decreased following estrogen treatment (Hewitt et al. 2003). Natural and synthetic estrogenic substances are known to exhibit characteristic degrees of activity. For example, long-acting estrogens such as estradiol ( $E_2$ ) or diethylstilbestrol (DES) elicit biological responses early (within 1-2 hours (h)) and persist to result in later responses (24-72 h) leading to maximal uterine growth. In contrast, short-acting estrogens, such as estriol ( $E_3$ ), match long-acting estrogens in eliciting the early responses, but due to their pharmacokinetics, do not persist and thus *in vivo* responses characteristic of later endpoints are blunted (Clark and Markaverich 1984; Katzenellenbogen 1984). However,  $E_3$  is able to match  $E_2$  in *in vitro* systems, such as its ability to stimulate MCF-7 cell growth, as culture conditions lack  $E_3$  metabolic clearing (Katzenellenbogen 1984). The xenoestrogens bisphenol A (BPA) and 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) elicited uterine transcription patterns similar to the proven short-acting estrogen  $E_3$  (Hewitt and Korach 2011; Klotz et al. 2000). Responses were highly correlated to those of  $E_2$  at 2 h, but attenuated and less correlated to  $E_2$  24 h (Hewitt and Korach 2011). We confirmed the short-acting estrogen-like activities of BPA and HPTE by observing uterine biological responses that were characteristic of short-acting estrogens. These endpoints included attenuated uterine weight increases after 72 h (3 days) and less induction of epithelial cell DNA synthesis after 24 h (Hewitt and Korach 2011). Recognizing not only the potential estrogenic activity of endocrine disrupting chemicals (EDCs) but also whether they have short-acting or sustained actions is important for evaluation of risks

to humans and wildlife. Due to their more sustained activity (Hewitt and Korach 2011; Safe et al. 2001), long-acting estrogens are more likely to impact estrogen sensitive tissues and thus might be considered of greater risk in terms of their impacts on exposed populations (Safe et al. 2001). In contrast, individuals that lack endogenous long-acting estrogens, including girls and postmenopausal women, might also exhibit susceptibility to short acting estrogens. It is therefore important to develop tools to predict not only estrogenicity but also the potential to elicit sustained responses. In the cancer field, biomarker panels of transcripts have been developed as tools for diagnosis and selection of treatments most likely to be effective (Gormley et al. 2007). Our approach aimed to utilize the well-characterized ovariectomized mouse uterus model to generate a screen that incorporates phenotypic estrogen-response endpoints and transcriptional biomarkers that distinguish estrogenic characteristics. We hope our approach will allow more detailed assessment of substances than *in vitro* high throughput screens, without undertaking extensive animal studies and comprehensive genomic analyses. Here, we describe development of panels of transcripts as biomarkers and phenotypic uterine responses to assess the activity of candidate compounds in terms of estrogenic activity and classification as short vs. long-acting estrogen. To this end, our uterine microarray datasets were analyzed using the EPIG tool (Extracting Patterns and Identifying co-expressed Genes) (Chou et al. 2007), and patterns characteristic of short-acting (only 2 h) or long-acting (both 2 h and 24 h) estrogen response were identified. To increase the power of validation of potential biomarker transcripts, we utilized biological endpoints including uterine weights, proliferation and/or apoptosis of epithelial cells, increase in luminal epithelial cell height and induction of apoptosis inhibitor, thus providing phenotypic anchoring to undergird transcriptional responses. Panels of 50 genes each at 2 or 24 h were identified using a combined analysis of variance (ANOVA) and principal component

analysis (PCA) approach, and validated by establishing activity of a candidate compound. These biomarkers and response endpoints (summarized in Table 1) will allow evaluation of potential estrogenic mechanisms for chemicals of environmental concern in a biological system. Diarylheptanoid (D3), a naturally phytoestrogen isolated from indigenous plant used in postmenopausal women in Thailand, was previously characterized to exhibit estrogen like activity in the uterus with a short-acting biological action compared to E<sub>2</sub> (Winuthayanon et al. 2009; Winuthayanon et al. 2013). Therefore, we employed D3 as a candidate to test our panel.

## **Methods**

### **Uterine bioassays**

Mice were housed 3-5 per cage in static micro-isolator solid-bottom cages (Lab Products Super Mouse 750™ cage) on autoclaved, hardwood bedding (Sani-chips, PJ Murphy, Montville, NJ) and maintained on a 12:12-h light:dark cycle at 22 ± 0.5 °C and relative humidity of 40% to 60%. Mice were provided ad libitum autoclaved rodent diet (NIH31, Zeigler Brothers, Gardners, PA) and given deionized water treated by reverse osmosis. All procedures were reviewed and approved by National Institute of Environmental Health Sciences Animal Care and Use Committee. All animals were housed, cared for, and used in compliance with the Guide for the Care and Use of Laboratory Animals in an AAALAC-accredited program. Animals were treated humanely and with regard for alleviation of suffering according to NIEHS Animal Care and Use Committee Guidelines and in Compliance with a NIEHS approved animal protocol. Ovariectomized adult (n=211, 8-10 weeks old) C57bl/6 mice were obtained from Charles River Laboratories (Raleigh NC), shipped to NIEHS facility and then housed 5 per cage for 10-14 days post-surgery to clear circulating ovarian hormones, before treatment by injection. Mice were assigned to treatment groups by cage mates, or in cases where fewer than 5 per group were used,

were divided by randomly removing some mice and combining them in a new cage. For 2 and 24 h samples (24h for weights, EdU incorporation and RT-PCR; 2h for RT-PCR), mice were injected with one of the following substances: 100  $\mu$ l sterile saline (0.85% NaCl; 5 mice) plus 0.1% ethanol, E<sub>2</sub> (4 mice 2h, 5 mice 24h), E<sub>3</sub> (4 mice 2h, 5 mice 24h) (Steraloids, or DES (3 mice 24h only, Sigma) diluted from a 1 mg/ml stock in 100% Ethanol into saline to a concentration of 250 ng per 100  $\mu$ l saline dose, 4,4',4''-(4-Propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol (PPT, 3 mice 24h only, Tocris Bioscience), diluted from a 50 mg/ml stock in 100% ethanol to a concentration of 250  $\mu$ g in 100  $\mu$ l saline + 10% Cremaphor EL (Sigma Chemicals). These treatments were all administered by intraperitoneal injections. The diarylheptanoid D3 was isolated and dosed as previously described (Suksamrarn et al. 2008; Winuthayanon et al. 2009; Winuthayanon et al. 2013). The vehicle for the D3 study (5 mice) was sesame oil plus 10% ethanol, D3 (4 mice 2h, 7 mice 24h) was diluted from a 250 mg/ml stock in 100% ethanol to a concentration of 2.5 mg in 100  $\mu$ l sesame oil (Sigma), and the E<sub>2</sub> solution for the D3 experiment (4 mice 2h, 7 mice 24h) was made by diluting a 1 mg/ml stock in 100% ethanol into sesame oil and adding ethanol to a concentration of 250 ng E<sub>2</sub> in 100  $\mu$ l of sesame oil plus 10% ethanol. These treatments were administered by subcutaneous injections. For all 24h experiments, all injections and collections were done in the morning. For 24h experiments, twenty two hours after hormone injection, 5-ethynyl-2'-deoxyuridine (EdU, 2 mg in 100  $\mu$ l PBS, Life Technologies) was injected intraperitoneally. Uterine tissue was collected 2 h later (24 h after hormone treatment). For 2h experiments, treatments were given without regard to time of day. For 72 h (3-day) endpoints tested compounds (vehicle 7 mice, E<sub>2</sub> 7 mice, E<sub>3</sub> 4 mice, DES 4 mice, PPT 4 mice, and for the D3 experiment vehicle 8 mice, E<sub>2</sub> 8 mice, D3 8 mice) were dissolved at the same concentrations as described above for 24 h endpoints, but all were in sesame oil (Sigma)

and injected subcutaneously each day for 3 days. Injections for 72 h experiments were initiated without regard for time of day, but were always 24 h apart. Uterine tissue was collected on the 4<sup>th</sup> day. For both 24h and 72h experiments, uterine tissue was weighed, a portion of one horn fixed in 10% Formalin (Fisher), and the remainder snap frozen in liquid nitrogen. For 2h experiments, uterine tissue was snap frozen in liquid nitrogen. Frozen uterine tissue was processed for RNA analysis by real time RT-PCR as previously described (Hewitt et al. 2003). Doses were selected based on minimal dose required to induce optimal uterine responses. (See Supplemental Material, Figure S1 for E<sub>2</sub>, DES, E<sub>3</sub>, and D3. PPT dose is based on responses reported in (Sinkevicius et al. 2008)). At least 3 animals per group were used based on previous studies indicating significant changes in endpoints are likely to be detected.

Fixed tissue was processed to detect incorporated 5-ethynyl-2'-deoxyuridine, (EdU) using the ClickIT Kit (Life Technologies) as previously described (Winuthayanon et al. 2010), or to indicate terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) positive apoptotic cells using the ApopTag Plus Peroxidase In Situ Apoptosis Kit (Cat# S7101, Millipore, Billerica, MA), also as described previously (Winuthayanon et al. 2010). Image analysis to evaluate cell proliferation and apoptosis was completed on transverse sections of EdU and TUNEL slides scanned with an Aperio Scanscope XT Digital Slide Scanner (Leica Biosystems, Buffalo Grove, IL) and viewed using Aperio® ImageScope v. 12.0.1.5027, (Aperio Technologies, Inc.). Percent positive EdU staining was determined by counting total and EdU Alexafluor 488 positive uterine luminal cells within the total uterine luminal area. Total uterine luminal area was selected by outlining the basement membrane of the uterine luminal epithelium. The percentage of luminal cells that were TUNEL positive was determined by counting the number of TUNEL positive per total epithelial cells in 6 regions of the uterine lumen (300-800

cells per slide). Luminal epithelial cell height (LEH) was measured in 5 areas per transverse section using the Ruler tool in ImageScope™. Data files were exported to Excel™ (v.14.3.9, Microsoft) and compiled into a single worksheet.

### **Microarray analyses**

Data from previous microarray studies were used for pattern analysis (Gene Expression Omnibus accession numbers GSE24525, GSE18168, GSE23072, GSE23241, GSE61921S; summarized in Tables S1 and S2). Data included: vehicle controls (11 samples) estradiol (E<sub>2</sub>, 13 samples), estriol (E<sub>3</sub>, 6 samples), DES (4 samples) and PPT (4 samples) for 2 or 24 h . All were from Agilent mouse 4x44K array chips (Agilent Technologies, Santa Clara CA), and data were acquired and analyzed as described previously (Hewitt et al. 2009; Hewitt et al. 2010; Hewitt and Korach 2011; Hewitt et al. 2012) .

### **Extracting patterns and identifying co-expressed genes (EPIG) analysis and deriving biomarker panels**

Details are described in Supplemental Material, “EPIG Analysis”. Briefly, batch corrected microarray datasets were analyzed using the Extracting Patterns and Identifying co-expressed Genes (EPIG) tool (Chou et al. 2007) resulting in 16 patterns (Figure 1 A-B). Fifty probes each (2 h and 24 h) that could distinguish estrogenic from non-estrogenic treatments (2 h) or long-acting from short-acting estrogens (24 h) were derived as described in Supplemental Material. Hierarchical clustering of the expression of the 50 2 h or 24 h probes with estrogens validated their selection (See Supplemental Material, Figure S2, Tables S3 and S4).

## **RT-PCR, Nanostring screen**

Probes to test the 2 h and 24 h biosets by RT-PCR were selected by first combining the 50 probe panels validated above (See Supplemental Material, Figure S2) with 26 additional 2 h probes or 12 additional 24 h probes covering RT-PCR primer sets for previously studied estrogen responsive transcripts, and were analyzed using Ingenuity Pathway Analysis. Probes that duplicated gene names and genes that represented the same biological function were consolidated. Genes for RT-PCR validation were then selected using a random number generator. For Nanostring analysis, 129 biomarker probes plus nine probes selected as housekeeping probes were submitted to Nanostring (Nanostring Technologies, Seattle, WA) for Code Set design, and RNA samples were shipped for processing and analysis. Briefly, 100 ng of each RNA sample was added to the Code Set in hybridization buffer and incubated at 65°C for 16 h. The Code Set consisted of Reporter and Capture probes that hybridize to the target sequences of interest, forming a tripartite complex. Assays were purified using the Prep Station (High Sensitivity Protocol), and data collected using the GEN2 Digital Analyzer, and raw counts were provided. Raw counts were then normalized using nSolver software (Nanostring) and exported into Partek Genomics Suite 6.6 (Partek Inc. St Louis MO) for clustering. Normalized values are shown in Supplemental Material, Table S5.

## **Statistical analysis for biological endpoints**

Real-time PCR, quantification of EdU and TUNEL positive cells and uterine epithelial cell height data represent mean  $\pm$  SEM. GraphPad Prism Software 6.0 for Windows was used for statistical analysis. Differences were considered significant when  $p < 0.05$  using ANOVA with Tukey multiple comparisons post-hoc, unless otherwise indicated.

## Results

### Biological endpoints

Proliferation of uterine epithelial cells, a hallmark of estrogenic response, was compared after E<sub>2</sub>, E<sub>3</sub>, DES (a synthetic estrogen), or PPT (a synthetic ER $\alpha$  selective agonist) treatment. All estrogens caused an increase in epithelial cells in S phase, as reflected by incorporation of the thymidine triphosphate analogue EdU (Figure 2A-B). At this 24 h time point, uterine wet weights were increased by E<sub>2</sub>, E<sub>3</sub> or DES ( $p < 0.05$ ), but not significantly by PPT (Figure 2C). However, after three days of treatment, E<sub>3</sub> did not significantly increase uterine weight, while E<sub>2</sub> or DES treatment led to a robust uterine weight increase ( $p < 0.0001$ ), whereas, PPT caused an increase ( $p < 0.001$ ) that was significantly attenuated compared to the increase induced by E<sub>2</sub> or DES ( $p < 0.001$ ) (Figure 2D). In the 72 h bioassay, E<sub>2</sub>, E<sub>3</sub>, DES and PPT increased the epithelial thickness and cell height ( $p < 0.001$ ; Figure 2E), however PPT and E<sub>3</sub> increased the epithelial thickness significantly less than E<sub>2</sub> ( $p < 0.001$  vs. E<sub>2</sub>; Figure 2E). Three days of administering E<sub>3</sub> did not induce transcripts of either the apoptosis inhibitor *Birc1a*, or the epithelial cell secretory protein *Ltf*, whereas E<sub>2</sub>, DES and PPT induced both of these (Figure 2F-G). In addition, E<sub>3</sub> and PPT exhibited significantly more TUNEL positive epithelial cells than E<sub>2</sub> or DES ( $p < 0.05$ ; Figure 2H-I) indicating increased apoptosis.

### Bioinformatic analysis to develop transcript panels

Towards our goal of developing panels of biomarker transcripts, which, in combination with these biological endpoints, might be used to screen for potential estrogenic activity of candidate chemicals, the EPIG tool (Extracting Patterns and Identifying co-expressed Genes) (Chou et al. 2007) was used to identify patterns of gene expression in our microarray datasets. As our datasets did not include sufficient numbers of test compounds for derivation of training,

validation, and test data sets to appropriately perform a prediction, we utilized a phenotypic anchoring approach. We correlated the gene expression according to the samples defined by the strength of estrogen response. The statistical framework of this correlation is well-described (Chou et al. 2007), and has statistical properties that are similar to an analysis of variance (ANOVA) model (sample variances), *t*-test (group comparisons) and signal processing (signal to noise thresholding). However, our approach also leverages the biological responses of the samples within a group, and correlation of gene expression between the groups, to extract patterns of genes which have a low probability of being detected by chance. Hence, the statistical rigor in our methodology supports the determination of these biomarker transcripts, when combined with these biological endpoints, as a potential screen for estrogenic activity of candidate chemicals (Table 1). Figure 1A shows profiles from vehicle, PPT, E<sub>3</sub>, DES, or E<sub>2</sub> after 2 h or 24 h. Each plot in Figure 1B shows one of the 16 response patterns extracted by EPIG. For example, estrogenic compounds (E<sub>3</sub>, PPT, E<sub>2</sub>, and DES) increased the levels of the 2481 probes in pattern 5 relative to vehicle (V) after 2 h, and the level of these probes returned to baseline by 24 h. Similarly, the estrogenic compounds decreased the levels of the 2002 probes in pattern 9 after 2 h. Probes in these patterns could thus be used to test whether a substance has estrogenic activity by evaluating the levels of these transcripts 2 h after treatment as all estrogens. E<sub>2</sub>, E<sub>3</sub>, PPT, and DES, increased or decreased levels of the probes in patterns 5 and 9, respectively in comparison to V. After 24 h. of treatment, E<sub>2</sub> and DES more robustly increased the 4467 probes in pattern 3 relative to V than PPT and E<sub>3</sub>. Only E<sub>2</sub> or DES, but not PPT or E<sub>3</sub>, increased the 691 probes in pattern 7. After 24 h of treatment, E<sub>2</sub> and DES repressed the 3311 probes in pattern 14 relative to V, while PPT and E<sub>3</sub> were less effective. Therefore, long vs. short-acting estrogens could be distinguished by evaluating the levels of transcripts from patterns 3, 7, or 14, 24 h after

treatment. After 2 h of treatment all the compounds increased the levels of the 2191 probes in pattern 1; however after 24 h only DES and E<sub>2</sub> maintained the increased levels of the probes, while the levels of the probes were lower after 24 h treatment with short-acting estrogens. After 2 h of treatment all the compounds decreased the levels of the 2751 probes in pattern 16; however after 24 h only DES and E<sub>2</sub> maintained the repressed levels of the probes, while the levels of the probes after 24 treatments with short-acting estrogens returned to the V level. Therefore, probes in patterns 1 and 16 could demonstrate estrogenic response at 2 h and also distinguish short-acting from long-acting estrogens at 24 h, as all estrogens increased (pattern 1) or decreased (patterns 16) the probes after 2 h, while only the long-acting estrogens maintained the response after 24 h (Figure 1B).

Two panels, each comprised of 50 probes, were selected using a combined ANOVA and PCA approach, detailed in the Supplemental Material, Methods section, “Deriving biomarker panels”. The first panel could be used to indicate potentially estrogenic substances based on their transcriptional response after 2 h that is distinct from V treated samples. The second panel could be used to indicate whether the estrogenic substance was short or long-acting, based on response at 24 h. Our previous work indicated BPA and HPTE are short-acting estrogens (Hewitt and Korach 2011). We used our previous microarray datasets (E<sub>2</sub>, E<sub>3</sub>, DES, BPA, HPTE, and PPT) to examine the expression of the probes in the biomarker panels. Supplemental Material, Figure S2A shows the level of the 50 probes 2 h after treatment with E<sub>2</sub>, E<sub>3</sub>, DES, BPA, HPTE or PPT relative to V (See Supplemental Material, Table S6); all compounds showed responses distinct from V with these probes in the 2 h panel. Supplemental Material, Figure S2B (See Supplemental Material, Table S7) shows the 50 probes selected to distinguish long-acting estrogens (E<sub>2</sub> and DES) from short-acting estrogens (E<sub>3</sub>, BPA, and HPTE). PPT was not included

in the short vs. long-acting 24 h panel analysis, as PPT is an ER $\alpha$  selective agonist, but has not been classified as short or long-acting. The biological data (Figure 2) indicated PPT exhibited a response intermediate between long and short-acting estrogens.

### **RT-PCR and validation of panels**

To validate our potential screening strategy, as detailed in Table 1, we evaluated a diarylheptanoid, (3R)-1,7-diphenyl-(4E,6E)-4,6-heptadien-3-ol or “D3”, from *Curcuma comosa* Roxb. (Zingiberaceae family; Figure 3A) (Suksamrarn et al. 2008), which has traditionally been used by women in Thailand to relieve post-menopausal symptoms. Studies have suggested this compound has estrogenic properties (Winuthayanon et al. 2009; Winuthayanon et al. 2013). As a test of the concept we utilized the biological response endpoints together with probes selected from each biomarker panel. D3 caused entry of uterine epithelial cells into S phase, as EdU incorporation and uterine weight was increased to a level comparable with E<sub>2</sub> after 24 h (Figure 3B-D). However, the uterine weight reached after 72 h was attenuated compared to the increase induced by E<sub>2</sub> (Figure 3D) and did not reach significance ( $p = 0.054$ ), as we previously reported (Winuthayanon et al. 2009; Winuthayanon et al. 2013). The attenuated weight increase after 72 h of treatment was reflected in increased TUNEL positive cells (Figure 3E-F) as well as attenuated LEH increase (Figure 3G). These biological observations are consistent with short-acting estrogenic activity. Therefore, we evaluated transcriptional responses of selected biomarker panel transcripts after 2 or 24 h of treatment (Figure 4A and B). Ten transcripts from the 2 h panel were tested, and 8 of the 10 transcripts showed similar responses with E<sub>2</sub>, E<sub>3</sub>, or D3, while D3-did not significantly induce two of the transcripts (*Cdkn1a* and *Stat5a*) and induced *Nup50*, less robustly than E<sub>2</sub> or E<sub>3</sub> (Figure 4A). Two of the transcripts selected from the 24 h panel (*Ndufab1*, and *Gfm1*) did not show any regulation with any of the tested substances (Figure 4B).

The remaining seven transcripts either showed no response with D3 (*Kifc2*, *Rorc*, *Sox4*, *Ccnb1*, *Nubp1* and *Aurkb*), or a response that was significant, but was blunted compared to the E<sub>2</sub> response (*Ccnb2*) (Figure 4B). Depending on the transcript, E<sub>3</sub> exhibited responses similar to D3 (*Kifc2*, *Rorc*, and *Sox4*) but in some cases was intermediate between D3 and E<sub>2</sub> (*Aurkb* and *Ccnb2*).

The RNA samples were then analyzed using a Nanostring Code Set that included 129 biomarkers and 9 housekeeping probes. Most of the probes in each of the panels confirmed the expected patterns, (See Supplemental Material, Figure S3), with the 2 h probes indicating regulation relative to vehicle by E<sub>2</sub>, E<sub>3</sub> or D3 (See Supplemental Material, Figure S3A), and the 24 h panel confirming differential regulation by long vs. short-acting estrogens (See Supplemental Material, Figure S3B).

## Discussion

Endocrine disruption describes a property of exogenous chemicals, either natural or man-made, that leads to perturbation of biological function via endogenous endocrine systems. These substances have the potential to greatly impact human health and the environment. However, endocrine disruption encompasses broad classes of chemicals that individually affect diverse biological signaling pathways. Thus, there is a need to focus on identifying which substances can impact which molecular events. In our study, we designed a multi-pronged approach for evaluation of chemicals suspected to affect estrogen signaling that combines transcriptional and phenotypic endpoints. As summarized in Table 1, a chemical can be used to treat ovariectomized mice, with uteri collected 2 h or 24 h after a single injection, or 72 h after the first of three daily injections. Substances that are estrogenic will increase epithelial cell DNA synthesis at the 24 h

endpoint, and will regulate transcripts in the 2 h panel relative to V or a non-estrogenic substance at the 2 h endpoint. Evaluation of uterine weights at the 24 h and 72 h endpoints can then be used to indicate whether an estrogenic substance is long-acting (weight increases observed) or short-acting (weight increase is attenuated or absent). At the 24 h endpoint, regulation of transcripts in the 24 h panel by long vs. short-acting estrogens will be distinct, as short-acting estrogens exhibit impeded regulation of these transcripts. Although the endpoints utilized in our approach are most relevant to uterine responses, the resulting classification of compounds as short or long-acting estrogens is important when evaluating potential impacts on other estrogen-sensitive tissues and species. Additionally, our approach specifically evaluated ER $\alpha$  mediated effects, as ER $\alpha$  is essential for mouse uterine responses, revealed by the full spectrum of estrogen responsiveness exhibited by ER $\beta$  null mice (Hewitt et al. 2003). Once substances are classified by this approach as short or long-acting estrogens, subsequent analyses could gauge ER $\alpha$  and ER $\beta$  mediated activities, as well as responses in other estrogen sensitive tissues and selective agonist vs. antagonist activities. Thus, further analyses subsequent to the screens we describe would be next steps to evaluate whether xenoestrogens behave as SERMs like tamoxifen (antagonistic in breast cells; agonist activity in endometrial tissue).

Our previous work uncovered a role for ER $\alpha$  in protecting epithelial cells from undergoing apoptosis subsequent to the initial proliferation response (Winuthayanon et al. 2010). Here, we demonstrate apoptosis of uterine epithelial cells as reflected in ineffective induction of the apoptosis inhibitor, *Birc1a*, and increased detection of TUNEL positive cells 72 h after treatment with short-acting estrogens (Table 1). This suggests that, similar to what we saw after ablation of epithelial ER $\alpha$  (Winuthayanon et al. 2010), short-acting estrogens are unable to maintain epithelial cell ER $\alpha$  activity following initial proliferation, and thus apoptosis occurs. This

selective ability of long-acting estrogens to induce *Birc1a* can be initially seen after 24 h (data not shown), but is most apparent after 72 h.

PPT is a known ER $\alpha$  selective agonist, but has not previously been classified as short or long-acting. Based on our observations in this study, some endpoints confirmed its estrogenic activity (DNA synthesis (Figure 2A), and 2 h transcript regulation (See Supplemental Material, Figure S2A)). However, other endpoints made it difficult to pin down the activity as short or long-acting; uterine weight did not increase after 24 h, but significant uterine weight increase occurred after 72 h. However, the increase was attenuated in comparison to long-acting estrogens (Figure 2D). Additionally, PPT was significantly less effective at inducing *Ltf* (Figure 2G) but induced the *Birc1a* apoptosis inhibitor equally well as long-acting estrogens (Figure 2F), which likely allows the observed weight increase after 72 h. *Birc1a* is not significantly increased by PPT after 24 h (not shown), and PPT treated uteri also exhibited a comparable amount of TUNEL positive cells as E<sub>3</sub> (Figure 2H-I). At 24 h PPT increases *Ltf* less effectively than E<sub>2</sub> or DES (not shown). Based on all the observed endpoints, we would classify PPT as an intermediate compound that is not a long-or short acting estrogen. We anticipate that other compounds tested in future analyses could exhibit intermediate characteristics as well, and this will be vital in predicting the risks of harmful effects, as less severe effects would be predicted for a shorter acting estrogen, but one with intermediate characteristics might need closer consideration.

Our present study using a diarylheptanoid (D3) compound isolated from a traditional Thai medicinal plant validated the approach outlined in Table 1. Most, but not all, transcripts from the biomarker panel probes tested by RT-PCR produced the expected transcript regulation patterns with E<sub>3</sub> or D3. A more comprehensive analysis using the Nanostring code set indicated our biomarker panel shows promise for distinguishing estrogenicity and relative strength of

endocrine disruptors. The regulation of three of the transcripts selected from the 24 h panel (*Nubp1*, *Ndufab1*, and *Gfm1*) could not be confirmed by RT-PCR. In the Nanostring analysis, *Nubp1* showed slight induction by E<sub>2</sub>, *Ndufab1* was minimally regulated while E<sub>2</sub> selective induction of *Gfm1* regulation was readily apparent (See Supplemental Material, Table S5). Differences may reflect disparities between the probes used on the microarray platform, Nanostring probes and the primer sets employed for RT-PCR.

Other approaches have focused on high throughput screens to identify potential endocrine disruptors, such as the ambitious Tox21 project at the National Toxicology Program. Tox21 aims to evaluate toxicants using robotic screens based on *in vitro* endpoints (Huang et al. 2011). Initial efforts include focus on nuclear receptor interactions utilizing ligand binding domains (LBD) of 10 different nuclear receptors in a Gal4-based response screen of a library containing approximately 3000 environmentally relevant chemicals (Huang et al. 2011). The objective of the Tox21 approach is a broad, high throughput initial evaluation to identify chemicals for further consideration. The approach we have outlined herein would be a logical next step for additional evaluation of chemicals positive in the Tox21 nuclear receptor assay that interact with ER $\alpha$ . The primary information obtained from the initial Tox21 screen reflects the chemical's ability to bind to and modulate the activity of the LBD of the ER $\alpha$ , as the assay included only this portion of the receptor, and measures regulation of an exogenous reporter gene activity. Such an approach lacks the inter- and intra- molecular interactions mediated in the context of the full-length ER $\alpha$  molecule and representation of *in vivo* responses of endogenous genes. In addition, the Tox21 assay will not capture subtleties of estrogenic response such as those typified by long vs. short-acting estrogenic activities. Our assay scheme is useful in the context of a "second step" after an initial broad screen, as the Tox21 approach is more realistic for taking on thousands of

chemicals. Our approach focuses on endpoints observed in mouse uterine tissue, and may not reflect effects in other estrogen target tissues such as breast or liver or impacts in other species. However, our panel of response is an appropriate partner to the broad Tox 21 approach and could be used to fine tune and finesse the mechanistic particulars and biological effects in a real whole animal context.

Moggs et al. have combined bioinformatics and phenotypic anchoring approaches to a similar model, the immature mouse uterus (Moggs et al. 2004). Although this approach is very useful towards defining transcriptional profiles that underlie phenotypic observations, it is not designed for the type of evaluation of mechanisms of action we focused on. Watanabe and colleagues also utilized the ovariectomized mouse model to compare transcriptional responses of uterine tissue 6 h after injection of E<sub>2</sub> or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Watanabe et al. 2004). In their study, some uterine transcripts were similarly regulated by E<sub>2</sub> or TCDD, suggesting estrogenic activity of TCDD. Many of the estrogen responsive transcripts showed a weaker response with TCDD. Unlike our approach, however, their screen was not a comprehensive attempt to select probes for screening. Another study employed the immature rat model to evaluate uterine transcript profiles 24 h after the last of 3 daily injections of E<sub>2</sub>, DES, or the endocrine disruptors BPA, genistein, and octyl-phenol or nonyl-phenol (Hong et al. 2006). Unlike our study, the transcript profiles for each substance was distinct, with few commonly regulated probes. This is especially surprising in the case of DES vs. E<sub>2</sub> regulated transcripts, which revealed only 126 common probes out of 555 E<sub>2</sub> regulated and 674 DES regulated probes, whereas we observed similar uterine responses to 72 h E<sub>2</sub> or DES in our model (See Figure 1). RT-PCR analysis of several transcripts revealed responses consistent with our observations, with E<sub>2</sub> and DES inducing transcripts at this late time point and BPA showing no induction (Hong et

al. 2006). Naciff and colleagues developed a gestational exposure model to evaluate uterine transcripts in gestational day 20 rat pups 2 h after the last daily injection of ethynyl E<sub>2</sub>, BPA, or genistein on gestational days 11-20 (Naciff et al. 2002). Their work develops transcriptional patterns reflecting transplacental exposure to estrogens. This approach is complementary to ours, with our focus on discerning potential modes of action reflecting biochemical characteristics and their efforts to develop a transcriptional profile “fingerprint” as evidence of gestational exposures.

*In vitro* models have also been developed utilizing Ishikawa cells, which are human endometrial cancer cell lines. Boehme et al. compared transcriptional profiles of ER $\alpha$  positive and negative Ishikawa cells dosed for 24 h with the estrogenic substances DES, BPA, Genistein, Zearalenone, Resveratrol, o,p'-Dichlordiphenyl-trichlorethane (DDT), or with the ER antagonist ICI 182 780 (Boehme et al. 2009). From their analysis, they derived a panel of estrogen responsive transcripts as candidates for estrogenic activity screening. Similar to our observations, BPA showed weaker activity. Naciff and colleagues compared transcriptional profiles of ER $\alpha$  positive Ishikawa cells treated with ethynyl E<sub>2</sub> or BPA for 8 h, 24 h or 48 h, with the focus of developing a relevant *in vitro* estrogen response model (Naciff et al. 2010). They identified 307 commonly regulated probes as candidate biomarkers of potential estrogenic activity. They further compared their BPA responsive Ishikawa transcripts to uterine transcriptional profiles from juvenile rats dosed with estrogen for 8 h, 24 h or 48 h and showed 362 commonly regulated transcripts (Naciff et al. 2010). Another approach reported transcriptional analysis on primary cultures of human endometrial endothelial cells (HEEC) 24 h after treatment with BPA, which leads to decreased HEEC growth (Bredhult et al. 2009). They did not observe effects on known estrogen responsive transcripts; however, HEECs are ER $\alpha$  negative and express ER $\beta$ , thus responses might reflect, in

part, ER $\beta$  targets. Abot and colleagues derived a panel of 40 estrogen responsive transcripts from published mouse uterus microarray datasets and also included evaluation of phenotypic endpoints, including uterine weights and the proliferative marker Ki67 (Abot et al. 2013). Their study was focused on the role of the AF1 domain of the ER $\alpha$  in the uterus, which has been shown to be an AF1 stimulated tissue (Arao et al. 2011). Abot et al. were able to use tamoxifen, an AF1 dependent estrogenic ligand, and mutations that disabled AF1 activity of the ER $\alpha$ , to demonstrate roles of AF1 as illustrated by changes in transcription or biological endpoints. These useful studies are not, however, designed to further screen mechanisms underlying activity or potential biological outcomes.

## **Conclusion**

We suggest that our proposed approach, outlined in Table 1, could be used to evaluate potentially estrogenic chemicals and also indicate possible biological mechanisms that would improve our understanding of the potential impacts on human health.

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**Table 1.** Scheme for screening potential estrogenic substances using phenotypic and transcriptional endpoints.

| Type of compound | 2 h<br>RNA panel <sup>a</sup> | 24 h<br>EdU <sup>a</sup> | 24 h<br>weight <sup>a</sup> | 72 h<br>weight <sup>b</sup> | 24 h<br>RNA Panel <sup>a</sup> | 72 h<br>TUNEL <sup>b</sup> | 72 h<br><i>Birc1a</i> <sup>b</sup> | 72 h<br><i>Ltf</i> <sup>b</sup> | 72 h<br>Epi Cell Height <sup>b</sup> |
|------------------|-------------------------------|--------------------------|-----------------------------|-----------------------------|--------------------------------|----------------------------|------------------------------------|---------------------------------|--------------------------------------|
| Non E            | -                             | -                        | -                           | -                           | -                              | -                          | -                                  | -                               | -                                    |
| Short-Acting E   | +                             | +                        | - / +                       | -/+                         | -/+                            | +                          | -/+                                | -/+                             | -/+                                  |
| Long-Acting E    | +                             | ++                       | +                           | ++                          | ++                             | -                          | ++                                 | ++                              | ++                                   |

= No response; -/+ = some or inconsistent response; + = moderate response; ++ = strong response; E; estrogen; Epi; epithelial.

<sup>a</sup>2h or 24h following a single injection. <sup>b</sup>72h after the first of 3 daily injections.

## Figure Legends

**Figure 1.** Microarray datasets analyzed by EPIG to extract expression patterns. **A.** Microarray datasets indicating co-regulation patterns. Includes V (24 h saline, 11 samples), E<sub>3</sub> 2 or 24 h (3 samples each), PPT 2 or 24 h (2 samples each), E<sub>2</sub> 2 h (5 samples) or 24 h (8 samples), and DES 2 or 24 h (2 samples each). Sixteen patterns were extracted, and are numbered across the bottom. Green boxes outline patterns 5 and 9, blue boxes outline patterns 3, 7, and 14, and red boxes outline patterns 1 and 16. **B.** Sixteen patterns extracted by EPIG. Inset describes organization of output. Each box represents one of the sixteen patterns. Inset in lower right summarizes the presentation of each box as follows: the pattern number is indicated (top center); the number of microarray probes within each pattern (n) is also indicated (top center). The Y axis is the normalized log ratio, with the vehicle baseline = 0 indicated by the red line. Each of the nine treatment groups is represented by the color coded dots as indicated in the legend. V (saline, red closed circle, 11 samples), E<sub>3</sub> 2 h and 24 h (green outlined circle and yellow outlined diamond, respectively, 3 samples each), PPT 2 h and 24 h (blue square and orange x, respectively, 2 samples each), E<sub>2</sub> 2 h (purple square, 5 samples) and 24 h (grey +, 8 samples), and DES 2 h and 24 h (light blue diamond and pink triangle, respectively, 2 samples each).

**Figure 2. Phenotypic endpoints of estrogenic response in uterus.** **A.** Incorporation of the TTP analogue, EdU (green signal). Blue signal indicates DNA using Hoescht. 24 h after administering an estrogenic substance (E<sub>2</sub>, E<sub>3</sub>, DES, or PPT), uterine epithelial cells show active DNA synthesis. Mice that received saline vehicle (V) have basal EdU incorporation. Bar = 0.1 mm. **B.** %EdU indicates quantification of EdU positive cells obtained from scans of slides as described in Methods section (mean ± SEM, n = 5-9 mice per group). \*\*  $p < 0.01$  compared to V by one way ANOVA with multiple comparisons and uncorrected Fisher's LSD. **C.** Uterine weights 24 h after injection of V (Saline), E<sub>2</sub>, E<sub>3</sub>, DES or PPT (mean ± SEM, n = 3-5 mice per group; \*\*\*\*, \*\*\*, \*  $p < 0.0001$ , 0.001, 0.05, respectively, compared to V, using one way ANOVA, with multiple comparisons, with Tukey's multiple test correction). **D.** 72 h (3 Day) Bioassay Weights of uteri collected 24 h after the last of three daily injections of V (sesame oil), E<sub>2</sub>, E<sub>3</sub>, DES or PPT (mean ± SEM, n = 4-7 mice per group). \*\*\*\*, \*\*  $p < 0.0001$ , 0.001, respectively, compared to V; PPT vs. DES or E<sub>2</sub> \*\*  $p < 0.001$ , using one way ANOVA, with multiple comparisons, with Tukey's multiple test correction. **E.** Luminal Epithelial Cell Height (LEH, μM), measured as

described in Methods (mean  $\pm$  SEM, n = 4-7 mice per group). (\*\*\*\*, \*\*\*  $p < 0.0001, 0.001$ , respectively, compared to V; +  $p < 0.0001$  vs. E<sub>2</sub> or DES, #  $p < 0.001$  vs. E<sub>2</sub> using one way ANOVA, with multiple comparisons, with Tukey's multiple test correction. **F.** 72 h: RT-PCR Analysis Level of RNA for apoptosis inhibitor, *Birc1a*. **G.** Secreted protein *Ltf* 24 h after the last of three daily injections of V (sesame oil), E<sub>2</sub>, E<sub>3</sub>, DES or PPT. (Mean  $\pm$  SEM n = 4-7 mice per group). \*\*\*\*, \*\*\*, \*\*  $p < 0.0001, 0.001, 0.01$  respectively, compared to V, +  $p < 0.01$  for PPT vs. E<sub>2</sub> using one-way ANOVA, with multiple comparisons, with Tukey's multiple test correction. **H.** Evaluation of apoptotic cells as indicated by TUNEL assay of uteri collected 24 h after the last of three daily injections of V (sesame oil), E<sub>2</sub>, E<sub>3</sub>, DES or PPT. Bar = 0.1 mm. **I.** % TUNEL positive luminal epithelial cells (mean  $\pm$  SEM, n = 4-10 mice per group) were measured as described in Methods. At  $p < 0.05$ , E<sub>2</sub> and DES (\*) differed from E<sub>3</sub> and PPT (+) using one way ANOVA, with multiple comparisons, with Tukey's multiple test correction. Arrow points to a TUNEL positive cell.

**Figure 3.** Responses to D3 consistent with short-acting estrogen activity. **A.** Structure of D3. **B.** EdU incorporation in uterine epithelial cells 24 h after treatment with V (saline), E<sub>2</sub> or D3; Bar = 0.1 mm. **C.** %EdU (Mean  $\pm$  SEM, n = 5 mice per group) was obtained from scans of slides as described in Methods section. \*\*\*  $p < 0.001$  compared to V using one way ANOVA with uncorrected Fisher's LSD. N = 5-7 mice per group. **D.** Uterine weights (Mean  $\pm$  SEM) 24 h after treatment with V (saline), E<sub>2</sub> or D3 (top; n = 9 mice per group) or 24 h after the last of 3 daily injections (bottom; n = 5 mice per group). \*\*\*, \*\*\*\*,  $p < 0.001, 0.0001$  respectively compared to V +  $p < 0.01$  compared to D3, using one way ANOVA with uncorrected Fisher's LSD. **E.** TUNEL assay of uteri collected 24 h after the last of three daily injections of V (sesame oil), E<sub>2</sub>, or D3 Bar = 0.2 mM. Arrow points to a TUNEL positive cell. **F.** %TUNEL positive cells (Mean  $\pm$  SEM, n = 8 mice per group) \*\*\*  $p < 0.001$  using unpaired t test. **G.** LEH ( $\mu$ M, Mean  $\pm$  SEM, n = 8 mice per group). \*\*\*\*  $p < 0.0001$  compared to V, +  $p < 0.001$  compared to E<sub>2</sub> using one way ANOVA with Tukey's multiple comparison test.

**Figure 4.** RT-PCR to validate Biomarker Panels. RNA samples from 2 or 24 h treatments with saline vehicle (V), E<sub>2</sub>, E<sub>3</sub> or D3. Values calculated relative to V and normalized to *Rpl7* (Mean  $\pm$  SEM, n=3-6 per group) \*\*\*\*, \*\*\*, \*\*, \*  $p < 0.0001, 0.001, 0.01, 0.05$ , respectively. +:  $p < 0.05$  vs. E<sub>2</sub> using one way ANOVA, with multiple comparisons, with Tukey's multiple test correction,

except for *Cdkn1a*, with no multiple test correction (Fisher LSD test). **A.** Transcripts selected from 2 h panel to indicate estrogenic response after 2 h. Cyclin-dependent kinase inhibitor 1A (*Cdkn1a*), Synaptopodin (*Synpo*), Cysteine rich protein 61 (*Cyr61*), Inhibin beta-B (*Inhbb*), Suppressor of cytokine signaling 3 (*Socs3*), Signal transducer and activator of transcription 5A (*Stat5a*), Thioredoxin interacting protein (*Txnip*), BTB (POZ) domain containing 10 (*Btbd10*), CDC42 small effector 1 (*Cdc42se*), and Nucleoporin 50 (*Nup50*). **B.** Transcripts selected from 24 h panel to distinguish long from short-acting estrogens after 24 h. Kinesin family member C2 (*Kifc2*), RAR-related orphan receptor gamma (*Rorc*), SRY-box containing gene 4 (*Sox4*), Aurkb aurora kinase B (*Aurkb*), Cyclin B1 (*Ccnb1*), Cyclin B2 (*Ccnb2*), nucleotide binding protein 1 (*Nubp1*), NADH dehydrogenase (ubiquinone) 1, alpha/beta subcomplex, 1 precursor (*Ndufab1*), and G elongation factor, mitochondrial 1 precursor (*Gfm1*).

Figure 1.

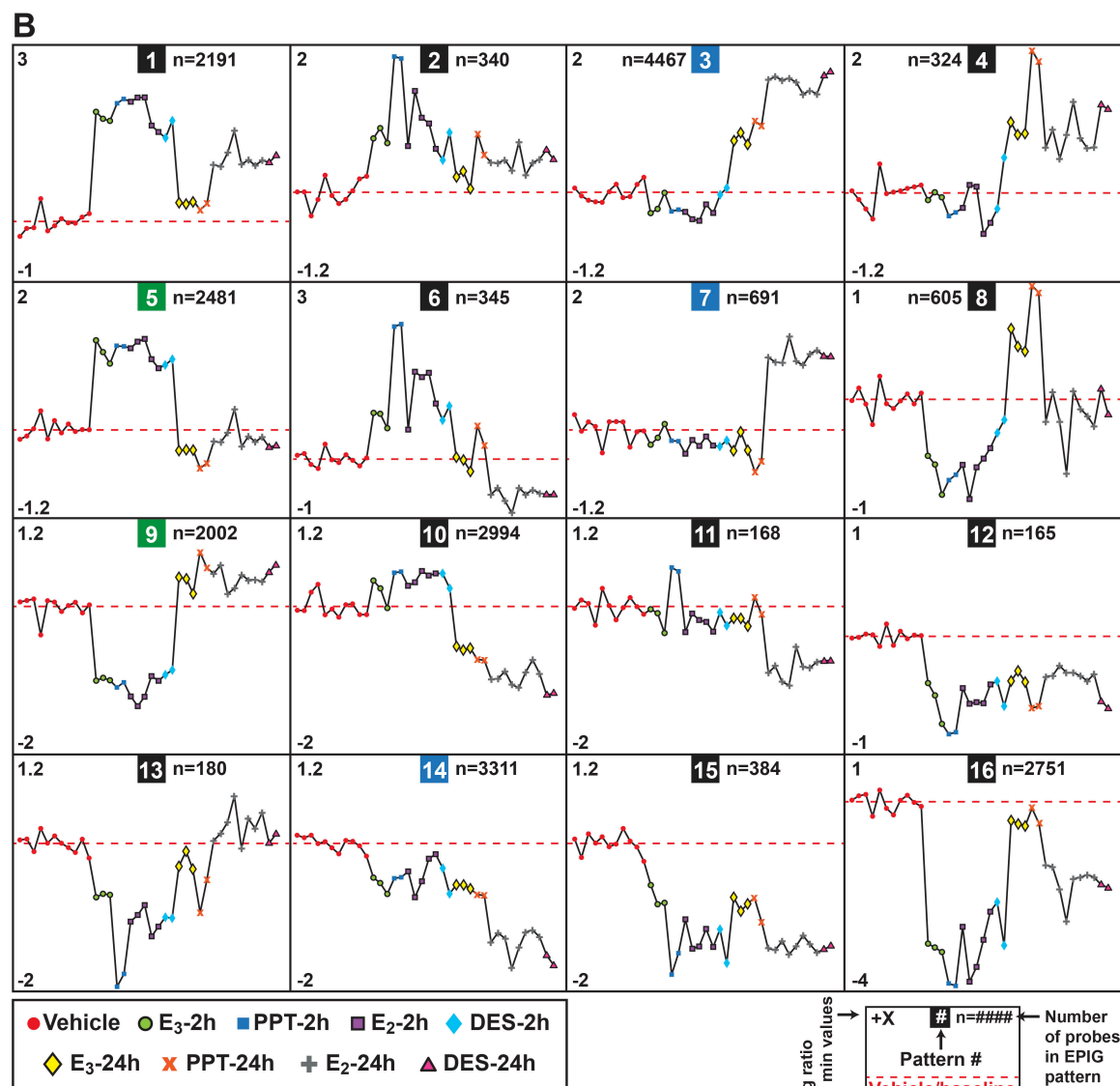
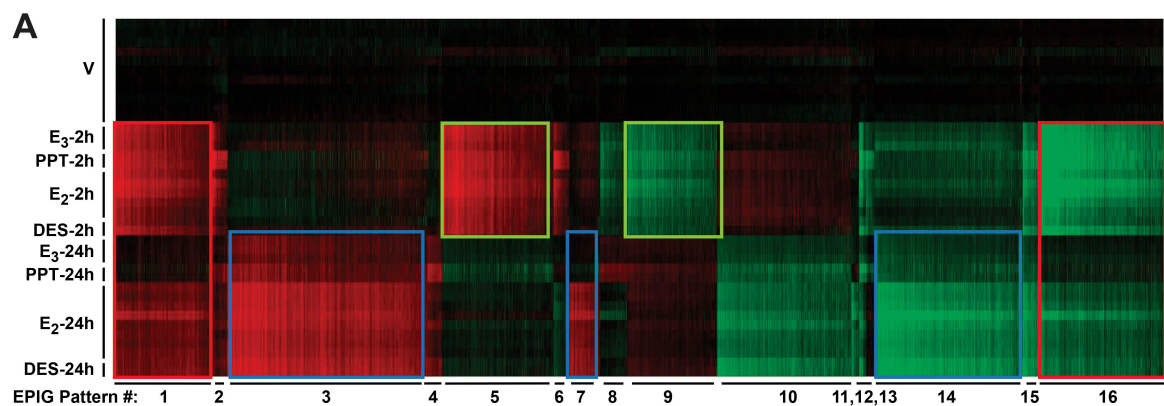
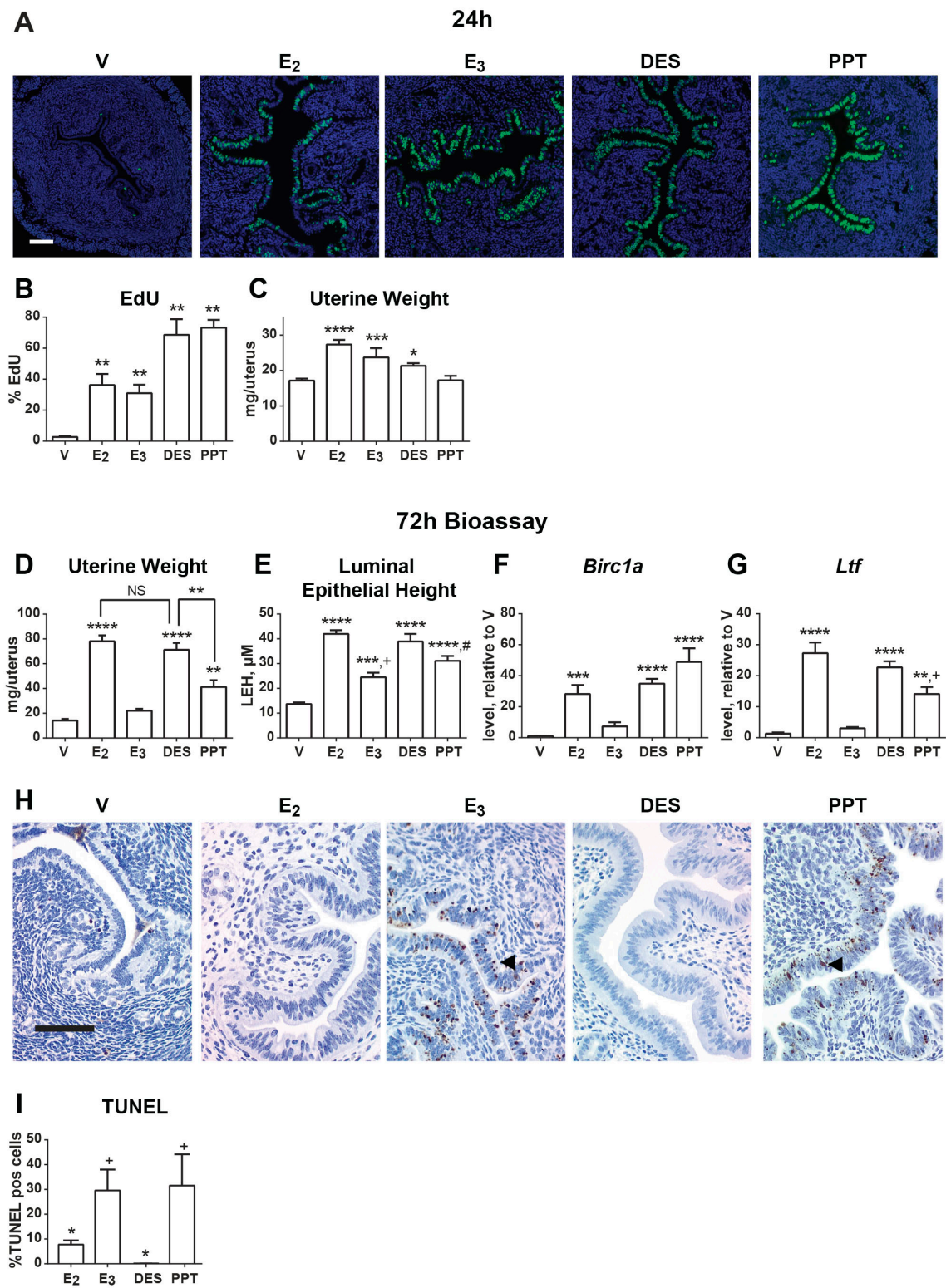
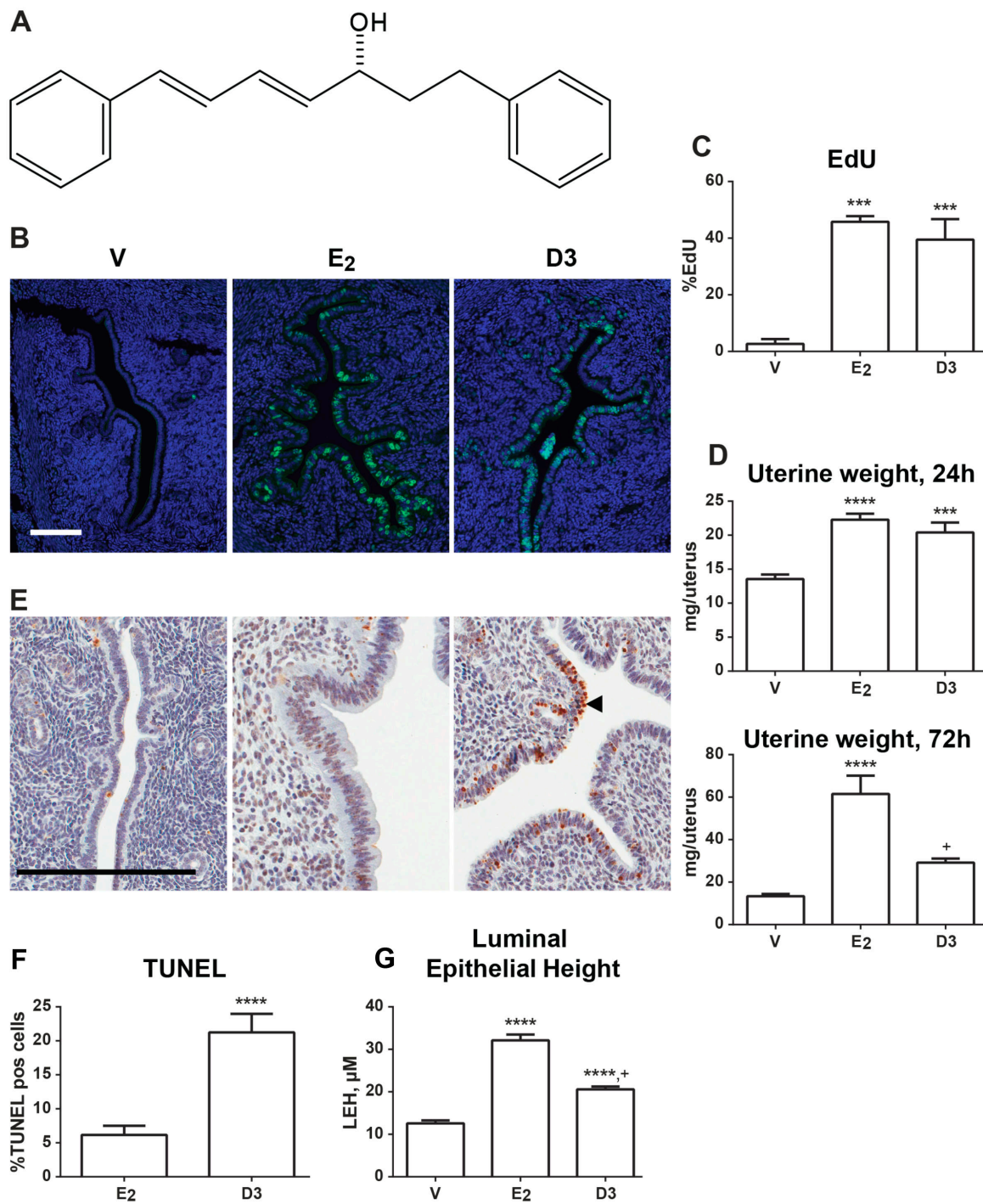


Figure 2.



**Figure 3.**



**Figure 4.**

